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Biochimica et Biophysica Acta 1345 (1997) 121–135

BBA

Review

The generation and actions of isoprostanes

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Received 28 May 1996; revised 25 October 1996; accepted 30 October 1996

Keywords: Isoprostane; Prostaglandin; Eicosanoid; Lipid peroxidation; Free radical

Contents

Abbreviations: IsoP(s), isoprostane(s); PG, prostaglandin; GC, gas chromatography; MS, mass spectrometry.

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PII S0005-2760(96)00162-2

1. Introduction

Isoprostanes (IsoPs) are prostaglandin (PG)-like compounds that are produced independent of the cyclooxygenase enzyme by free radical catalyzed peroxidation of arachidonic acid. There are a myriad of compounds formed as products of free radical catalyzed lipid peroxidation. The notion that PG-like compounds could be generated in vitro non-enzymatically as products of autoxidation of fatty acids was actually first demonstrated over 20 years ago [1,2]. However, this interesting observation was never carried beyond the realm of an in vitro chemical curiosity of fatty acid autoxidation nor was it ever explored whether such products could be generated by free radical catalyzed peroxidation of lipids in a biological fluid in vitro.

2. Discovery of F_2-IsoPs as products of autoxidation of lipids in biological fluids in vitro

Previously we demonstrated that PGD_2 is predominantly metabolized in vivo in humans by a 11-ketoreductase pathway to form 9α,11β-PGF_2 [3]. We also found that PGD_2 is very unstable under certain conditions and can readily undergo isomerization of the lower side chain prior to reduction of the carbonyl group by 11-ketoreductase, yielding isomers of 9α,11β-PGF_2 [4]. During the course of attempting to detect these isomeric F-ring metabolites of PGD_2 in human plasma and urine using a gas chromatography (GC) mass spectrometric (MS) method of assay, a puzzling observation was made. In freshly obtained plasma samples from normal volunteers that were processed and analyzed immediately, a series of peaks were detected with characteristics of F-ring PGs. However, when plasma that had been stored for several months at −20°C was analyzed, the same peaks were detected as in fresh plasma but at levels approx. 100-fold higher [5]. A series of investigations undertaken in an attempt to explain this phenomenon led to the discovery that these compounds were PGF_2-like compounds that were generated non-enzymatically by autoxidation of plasma arachidonic acid during storage [5].

3. Mechanism of formation

The mechanism envisioned for the formation of these compounds is shown in Fig. 1. Abstraction of bis-allylic hydrogens of arachidonic acid by free radicals leads to the formation of the three arachidonoyl radicals as shown. Subsequent attack by O_2 results in the formation of four peroxyl radical derivatives of arachidonic acid. These peroxyl radical derivatives then undergo endocyclization followed by further addition of O_2 to form PGG_2-like bycyloendoperoxides. Reduction of these PGG_2-like compounds results in the formation of PGF_2-like compounds. Depending on the location of the peroxyl radical derivatives of arachidonic acid, four regioisomers are formed as noted (I–IV). Each of these regioisomers can be theoretically comprised of eight racemic diastereomers. Thus, a multiplicity of compounds can be generated by this process, although the formation of some is likely favored over others. Because these

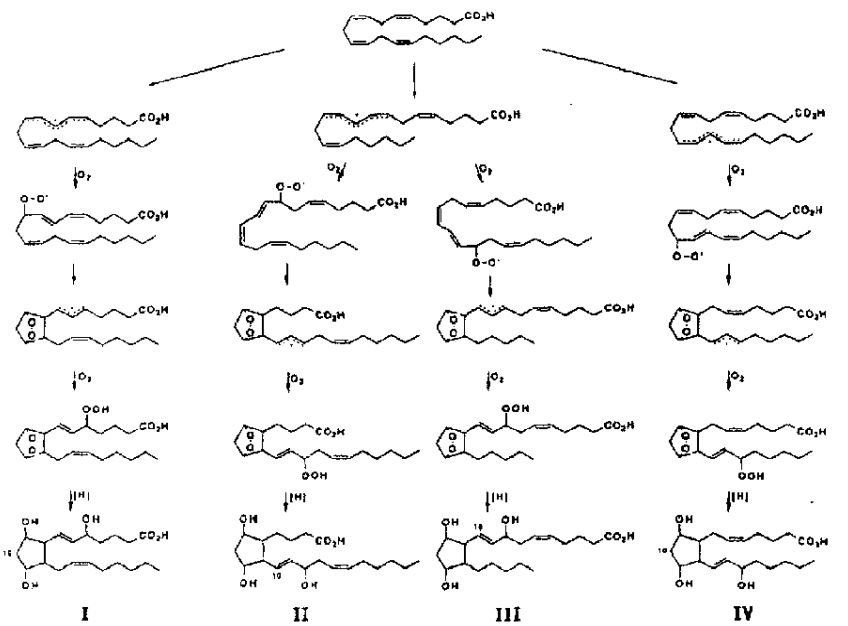

Fig. 1. Mechanism of formation of the F_2-IsoPs. This pathway leads the formation of four regioisomers (I–IV). For simplicity, stereochemical orientation is not indicated. Each regioisomer theoretically is comprised of a mixture of eight racemic diastereomers. Reprinted with permission from Academic Press.

compounds contain the F-type prostane ring and are isomeric to $PGF_{2\alpha}$ derived from the cyclooxygenase enzyme, they have been termed F_2-IsoPs.

There are some structural aspects of F_2-IsoPs that are worthy of mention. Owing to the fact that the F-ring compounds derive from the reduction of endoperoxide intermediates, the prostane ring hydroxyls must be oriented *cis*, although they can be oriented either α,α or β,β. Another structural feature that contrasts with that of cyclooxygenase-derived PGs relates to the stereochemistry of the side chains. It has been shown that the side chains of PG-like compounds generated by autoxidation of fatty acids are predominantly oriented *cis* in relation to the prostane ring [6].

4. Discovery of the formation of F_2-IsoPs in vivo

Impressed with the facility with which F_2-IsoPs are formed in vitro, e.g., large quantities can be generated even at $-20°C$ during storage of plasma, we explored the more important question, namely are IsoPs also produced in vivo? Individual GC peaks representing one or more of these compounds can be detected in fresh plasma from normal volunteers that is processed and analyzed immediately without storage at levels in the range of 35.5 ± 6.1 pg/ml (mean $\pm$ 1 S.D.). From the above discussions, it can be understood that one of our initial concerns was whether the levels detected reflected true endogenous circulating levels of F_2-IsoPs, or whether they were formed ex vivo by autoxidation of plasma lipids. The possibility that the levels detected in fresh plasma arose from rapid autoxidation ex vivo seemed unlikely because plasma contains antioxidants and we found that drawing blood into syringes containing the antioxidant butylated hydroxytoluene, or the reducing substance triphenylphosphine, failed to reduce levels measured [7]. In addition, we found that the levels quantified in urine from normal volunteers, which are quite high (1.6 ± 0.6 ng/mg creatinine), were not

increased by incubation of urine at 37°C for 1 week. This indicated that these compounds cannot be generated by autoxidation in urine, which is understandable because urine contains only small quantities of lipid. Convincing evidence that F_2-IsoPs are, in fact, formed in vivo was obtained when it was demonstrated that levels of these compounds detected in plasma obtained from rats that were administered CCl_4 or the herbicide diquat to induce severe free radical injury in vivo were increased by as much as 200-fold above levels measured in control rats [7].

There is another interesting aspect related to the finding that IsoPs are formed in vivo. It is well established that only trivial amounts of arachidonic acid are present in the unesterified state; the vast majority exists esterified to phospholipids. In view of this, we explored the possibility that F_2-IsoPs are initially formed in situ esterified to phospholipids and then subsequently released in free form by phospholipases [8]. This was an intriguing question because it counters the dogma that prostanoids do not exist esterified to phospholipids. To gain support for the notion that IsoPs are initially formed in situ on phospholipids and subsequently released in free form, we correlated the time-course of appearance of increases in levels of F_2-IsoPs esterified in liver phosholipids and free in the circulation following administration of CCl_4 to rats to induce lipid peroxidation. We found that levels of esterified F_2-IsoPs increased rapidly, reaching approx. one-half maximum as early as 15 min; whereas the appearance of free F_2-IsoPs in the circulation was delayed considerably [9]. To obtain direct physical evidence for the presence of F_2-IsoPs esterified to phospholipids, we administered CCl_4 to rats to induce intense lipid peroxidation and then subjected a lipid extract from the liver to normal phase HPLC purification using a system that primarily separates phosphatidylcholine species from less polar lipids [8]. Fractions collected were then subjected to base hydrolysis and analyzed for free F_2-IsoPs to detect fractions that may contain esterified F_2-IsoPs. We found that fractions that appeared to contain esterified F_2-IsoPs eluted in a region that was more polar than unoxidized phosphatidylcholine. These fractions were then analyzed directly by fast atom bombardment mass spectrometry (MS) which unambiguously identified phosphatidylcholine species with palmitate or stearate esterified at the $sn-1$ position and an F_2-IP esterified at the $sn-2$ position [8]. Subsequently, a more detailed analysis of phospholipid-containing F_2-IsoPs was carried out using collision-induced dissociation tandem MS [10].

An interesting question, however, remains regarding the mechanism involved in the hydrolysis of IsoPs from phospholipids in vivo. It is reasonable to assume that the hydrolysis is catalyzed by phopholipases. In the in vitro situation, we have found that bee venom phospholipase A_2 effectively hydrolyzes IsoPs from lipids [8]. However, the nature of the phospholipase(s) responsible for the hydrolysis of IsoPs in vivo remains to be established.

After determining that IsoPs are initially formed by peroxidation of arachidonic acid esterified to tissue lipids, we have analyzed a variety of normal animal tissues for levels of esterified F_2-IsoPs, including liver, heart, brain, skeletal muscle, aorta tissue, ocular lens, kidney, and lung and found detectable levels in all of these tissues [9,11]. Analysis of human tissues has been limited to gastric biopsies, where levels of F_2-IsoPs were also present in detectable quantities (unpublished data). These findings are consistent with the fact that detectable levels of unesterified F_2-IsoPs are present in all normal biological fluids from both animals and humans.

5. Discovery of E_2- and D_2-IsoPs and isothromboxanes

The cyclooxygenase-derived endoperoxide intermediate, PGH_2, is unstable and rearranges in aqueous buffer to PGD_2 and PGE_2 with a $t_{1/2}$ of several minutes [12]. Thus, it was reasonable to suspect that the intermediate IsoP endoperoxides, if not efficiently reduced to F_2-IsoPs, may also rearrange to form E- and D-ring IsoPs (Fig. 2). A series of experiments were carried out to explore this possibility which convincingly demonstrated that E_2/D_2-IsoPs are in fact also formed in vivo [13]. Levels of E_2/D_2-IsoPs esterified in a variety of tissues of the rat were found to be approximately one-third the levels of F_2-IsoPs. However, in contrast to F_2-IsoPs, E_2/D_2-IsoPs cannot be detected in the circulation of humans or rats under normal circumstances, although they can be detected in the circulation of rats that have been

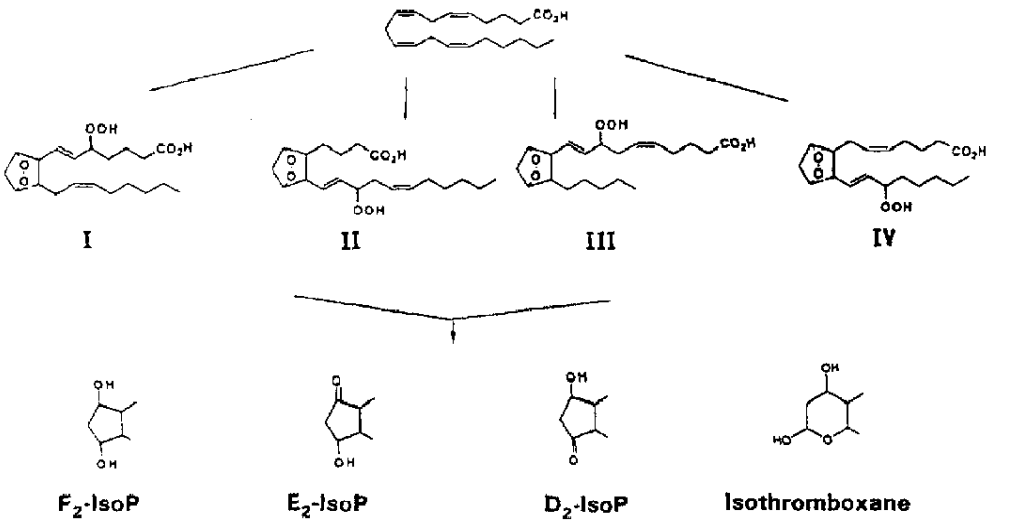

Fig. 2. Potential fates of the bicycloendoperoxide intermediates of the isoprostanes (I–IV) derived from the peroxidation of arachidonic acid. The endoperoxides can either undergo reduction to F_2-IsoPs or rearrangement to E_2/D_2-IsoPs and isothromboxanes.

administered CCl_4 to induce severe lipid peroxidation. The reason for this is unclear but could be explained if there are differences in the rate of the metabolic clearance of E_2/D_2- and F_2-IsoPs.

It has previously been shown that the cyclooxygenase-derived endoperoxide, PGH_2, can also rearrange nonenzymatically to form small quantities of thromboxane A_2, which can be catalyzed by iron [14]. Therefore, we also explored whether the IsoP endoperoxides may also rearrange to form isothromboxane compounds in vivo and have provided convincing evidence that this does occur [15]. Levels of isothromboxanes esterified to tissue lipids, e.g. in liver, are similar to the levels of E_2/D_2-IsoPs.

6. Importance of the discovery of the formation of IsoPs

The discovery of the formation of IsoPs encompasses several important areas. First, the finding that F_2-IsoPs can be generated in biological fluids in vitro, even at a reduced temperature of $-20°C$, presaged potential important analytical ramifications for the analysis of prostanoids [5]. This concern applies to both physical and immunological methods of analysis. Particular precautions must be taken to avoid generation of IsoPs in lipid-containing biological fluids prior to analysis (vide infra). These compounds have similar chromatographic properties on both TLC, HPLC, and GC as $PGF_{2\alpha}$ and thus could confound an interpretation of whether a PGF_2 compound measured by physical methods, e.g. GC/MS, was enzymatically or non-enzymatically generated. In addition, antibodies used in immunoassays for cyclooxygenase-derived $PGF_{2\alpha}$ may potentially cross-react to a significant extent with F_2-IsoPs. For example, we have found that an antibody obtained commercially (Amersham Life Science) to the PGD_2 metabolite, $9\alpha,11\beta$-PGF_2, exhibits considerable cross-reactivity with the complex mixture of F_2-IsoPs, even though the prostane ring hydroxyls in F_2-IsoPs are oriented *cis* [5]. Interesting also is a report that a PGE_2 antibody cross-reacts to a significant extent with F_2-IsoPs [16].

Another important aspect of the discovery of IsoPs relates to the use of measurement of IsoPs as an index of lipid peroxidation and oxidant stress in vivo. It has been well recognized for a long time that one

of the greatest needs in the free radical field is the availability of a reliable non-invasive method to assess oxidative stress status in vivo in humans [17]. This is because most methods previously available to assess oxidant stress in vivo suffer from a lack of specificity and/or sensitivity and are unreliable [17]. However, as discussed subsequently, a substantial body of evidence has been obtained which indicates that measurement of IsoPs, i.e. in urine and plasma, provides a reliable non-invasive approach to assess lipid peroxidation in vivo and, as such, represents what appears to be a major advance in our ability to assess oxidative stress status in humans. Further, the sensitivity of the mass spectrometric method of analysis appears sufficient to quantify levels of F_2-IsoPs in small biopsies of human tissue, e.g. gastric biopsies (unpublished data), which should permit an assessment of oxidant injury in key human tissues of interest, if amenable to biopsy.

Thus, the ability to quantify F_2-IsoPs opens up countless new avenues for investigation to explore the role of free radicals in the pathophysiology of a wide range of human diseases. It also provides an extremely valuable tool to define the clinical pharmacology of antioxidant agents. There are numerous large trials either planned or underway examining the effect of antioxidants, e.g. vitamin C, vitamin E, to prevent or ameliorate some of the pathology of diseases in which free radicals have been implicated, e.g. atherosclerosis. However, such studies are hampered by insufficient information regarding what doses and combinations of antioxidants are maximally effective. Measurement of IsoPs should provide a valuable approach to define the clinical pharmacology of antioxidants. In support of this, the formation of F_2-IsoPs has been found to increase significantly in animals rendered deficient in vitamin E, even in the absence of the administration of an agent to induce endogenous oxidant injury [18]. In addition, administration of antioxidants has been shown to inhibit the formation of IsoPs in animal models of oxidant injury [19]. Further, we have found that the administration of a combination of antioxidants at high doses (4 g/d vitamin C, 3200 IU/d synthetic vitamin E, and 300 mg/d natural β-carotene) to normal volunteers for a period of two weeks inhibited the formation of F_2-IsoPs esterified to plasma lipids by approximately 25–55% (unpublished data). It is important to emphasize that the magnitude of inhibition of F_2-IsoP formation that occurred was likely not maximal owing to the fact that the antioxidants were only administered for a short time (2 weeks), which is likely an insufficient duration of time for the lipophilic antioxidants to reach steady-state [20]. We have also examined the effect of administering 400 IU and 800 IU/d of vitamin E (α-tocopherol) for two weeks on plasma concentrations of unesterified F_2-IsoPs. In the group given 400 IU/d vitamin E, circulating concentrations of F_2-IsoPs decreased 25%, but this did not reach statistical significance. However, in the group taking 800 IU/d of vitamin E, plasma levels of F_2-IsoPs fell by 37%, an effect that was highly significant ($p = 0.007$) (unpublished data). These data suggest that measurement of IsoPs can be used to quantitatively define the pharmacodynamic effects of antioxidants to inhibit free radical processes in vivo in humans. Such information in the latter group would contribute in a valuable way to long-term studies aimed at exploring the efficacy of antioxidants to prevent the development of pathology, e.g. atherosclerosis.

It is of considerable interest that the levels of IsoPs in normal human plasma and urine exceed levels of cyclooxygenase-derived PGs and thromboxane by more than an order of magnitude. Although this clearly indicates that the formation of IsoPs is a major pathway of arachidonic acid disposition, it is important to consider the relevance of the finding that levels of F_2-IsoPs are sufficient to be detected in every normal biological fluid that has been assayed; this includes plasma, urine, cerebrospinal fluid, bile, and gastric juice. Previously, using other methods to assess lipid peroxidation, there had been little concrete evidence obtained indicating that lipid peroxidation occurs in vivo except under abnormal circumstances of marked oxidative stress. However, the finding of detectable levels of F_2-IsoPs in all normal animal and human biological fluids and esterified in normal animal tissues indicates that there is certain level of ongoing lipid peroxidation that is incompletely suppressed by antioxidant defenses, even in the normal state. This finding may lend some support to hypothesis that the normal aging process is due to enhanced oxidant damage of important biological molecules over time [21]. In fact, there is also some evidence suggesting that there is a trend for the

formation of F_2-IsoPs to increase with increasing age in humans [22].

Another very important aspect of the discovery of IsoPs has been the finding that at least two IsoPs, 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$, can exert potent biological activity (vide infra). Thus, these compounds may not simply be markers of lipid peroxidation, but may also participate as mediators of oxidant injury. The possibility that additional IsoPs will also be found to possess biological activity awaits the availability of additional synthetic compounds. The chemical synthesis of additional IsoPs is currently being pursued by at least three laboratories. Regarding the biological activity of isothromboxanes, one might anticipate that, analogous to cyclooxygenase-derived thromboxane, only the compounds with a thromboxane A_2-like ring, but not a thromboxane B_2-like ring structure, would be bioactive. Unfortunately, because of the marked instability of the thromboxane A_2 ring, it will be difficult, if not impossible, to synthesize isothromboxane A_2 compounds for purposes of biological testing.

7. Method of analysis of F_2-IsoPs

The method that we have employed for measurement of F_2-IsoPs is a negative chemical ionization GC/MS assay [11]. This method is highly sensitive with a lower limit of detection in the low picogram range and is highly accurate (precision = ±6%, accuracy = 96%). Previously we have used commercially available $[^2H_4]PGF_{2\alpha}$ but recently 8-iso-$[^2H_4]PGF_{2\alpha}$, one of the more abundant F_2-IsoPs produced in vivo [23], has become available commercially (Cayman Chemical, Ann Arbor, MI) and may be the preferred deuterated internal standard for measurement of F_2-IsoPs. Measurement of esterified levels of F_2-IsoPs in tissues is accomplished by measurement of free compounds following alkaline hydrolysis of a lipid extract of tissue [11].

However, the mass spectrometric method of assay is labor intensive and the technology is not widely available in the scientific community, primarily because mass spectrometers are expensive and costly to maintain. However, both commercial enterprises and academic investigators are developing immunoassays for specific F_2-IsoPs [24], which should greatly expand research in this area. Recently a method was also reported for measurement of 8-iso-$PGF_{2\alpha}$ using an immuno affinity column for purification coupled with quantitation by mass spectrometry [25].

7.1. Precautions associated with the measurement of IsoPs

As discussed previously, the original discovery of F_2-IsoPs evolved as a result of autoxidation of plasma arachidonic acid during storage at −20°C [5]. Thus, precautions must be taken to prevent artifactual generation of IsoPs by autoxidation in samples that are analyzed for IsoPs both during storage and during sample processing. Whereas we found that IsoPs are generated by autoxidation in lipid-containing samples during storage at −20°C, we have found that autoxidation does not occur in lipid containing samples, e.g. plasma, that are initially snap frozen in liquid nitrogen and stored at −70°C for up to six months. However, once thawed, samples should be assayed immediately and not refrozen and stored. Tissue samples collected for measurement of esterified IsoPs also must either be analyzed immediately or snap frozen in liquid nitrogen and stored at −70°C. Autoxidation is not a problem with urine samples owing to the fact that urine contains only trivial quantities of lipid; we have found that levels of F_2-IsoPs do not increase even when urine is incubated at 37°C for one week.

In addition to precautions to prevent autoxidation of samples that are stored, similar precautions are required during processing of certain types of samples, namely addition of antioxidants. Measurement of free unesterified levels of IsoPs in biological fluids, even those that contain lipid, does not require the addition of antioxidants. For example, in plasma, the lipoproteins are not retained by the initial C-18 cartridge extraction and any free phospholipids, etc. in plasma bind tightly to plasma albumin and also are not retained. Thus, after the initial C-18 cartridge extraction, the IsoP-containing extract can also be safely stored without concerns of autoxidation. Where autoxidation is of primary concern is with the analysis of esterified IsoPs in tissues and, particularly, plasma lipoproteins. This is likely due to the more

extensive manipulation required for analysis of esterified IsoPs. These samples first require a Folch lipid extraction followed by evaporation under N_2 of a large volume of organic solvent. Then the samples must be subjected either to base hydrolysis, in the case of F_2-IsoPs, or enzymatic liberation of E_2/D_2-IsoPs with bee venom phospholipase A_2, after which free compounds are extracted using a C-18 cartridge. We have found that the addition of 0.005% butylated hydroxytoluene (BHT) to the organic phase during the Folch lipid extract effectively prevents artifactual generation of IsoPs by autoxidation in tissue samples [11]. For some reason, however, we have found that autoxidation occurs more readily when IsoPs esterified to plasma lipoproteins are measured. In this case, addition of BHT does not effectively prevent autoxidation completely. However, if the reducing agent, triphenylphosphine (0.5%) is also added to the organic phase along with BHT, autoxidation during processing of plasma lipoproteins can be effectively suppressed [26].

An attractive approach for assessing total endogenous F_2-IsoP production is measurement of F_2-IsoP metabolites [27]. Measurement of metabolites of F_2-IsoPs in urine has the advantage not only of circumventing the problem of artifactual generation of F_2-IsoPs ex vivo by autoxidation but can also provide an integrated assessment of F_2-IsoP production over time. It has been shown that unmetabolized cyclooxygenase-derived prostaglandins in urine derive almost exclusively from their local formation in the kidney [28]. Although at present we do not know whether urinary unmetabolized F_2-Isops derive from the kidney, it is reasonable to suspect, that at least in part, this is the case. In this regard, we found that there was a very high correlation between the urinary excretion of F_2-IsoP metabolites and circulating concentrations of F_2-IsoPs under circumstances associated with mild/moderate overproduction of F_2-IsoPs in humans ($r = 0.97$, $P < 0.001$) [29]. In contrast, there was a very poor correlation between the urinary excretion of unmetabolized F_2-IsoPs and plasma levels of F_2-IsoPs ($r = 0.29$, $P = 0.41$) (unpublished data). Thus, although the levels of unmetabolized F_2-IsoPs in urine may increase when the production of F_2-IsoPs from extrarenal sources is enhanced due to filtration of F_2-IsoPs from the circulation, measurement of unmetabolized urinary F_2-IsoPs may not be as sensitive to modest increases in systemic production of IsoPs as is measurement of circulating concentrations of F_2-IsoPs or F_2-IsoP metabolites.

8. Metabolism of IsoPs

Our knowledge of the metabolic fate and kinetics of metabolic disposition of IsoPs is somewhat limited. We have carried out an experiment in the rat exploring the time-course of disappearance of 8-iso-$PGF_{2\alpha}$ from the circulation. In this experiment, 8-iso-$PGF_{2\alpha}$ was infused intravenously, the infusion was discontinued, and blood samples were obtained at various time points and the plasma concentration of 8-iso-$PGF_{2\alpha}$ measured [9]. From the data obtained it was determined that the $t_{1/2}$ of the clearance of 8-iso-$PGF_{2\alpha}$ from the circulation was ≈ 16 min. Although not examined directly, it is likely, analogous to the metabolism of other prostanoids, that the lung is a major site of metabolic clearance of F_2-IsoPs from the circulation. This notion is supported by the finding that the creation of a portal caval shunt and ligation of the hepatic artery in rats, completely eliminating clearance of 8-iso-$PGF_{2\alpha}$ by the liver, only prolonged the $t_{1/2}$ of the clearance of 8-iso-$PGF_{2\alpha}$ from the circulation from 16 to 21 min [9].

Recently, we explored the metabolic fate of 8-iso-$PGF_{2\alpha}$ in humans using radiolabelled 8-iso-$PGF_{2\alpha}$ [30]. Interestingly, $\approx 43\%$ of excreted radioactivity was unextractable into ethyl acetate, suggesting the presence of very polar material, perhaps polar conjugates [31]. The major urinary metabolite of 8-iso-$PGF_{2\alpha}$ was identified as 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. This metabolite represented 29% of the total extractable recovered radioactivity in urine. It is interesting that the Δ^5 double bond in this metabolite had been reduced since reduction of the Δ^5 bond is not a prominent feature of the metabolism of cyclooxygenase-derived eicosanoids [32]. Although speculative, perhaps the inversion of the stereochemistry of the upper side chain renders 8-iso-$PGF_{2\alpha}$ or 2,3-dinor-8-iso-$PGF_{2\alpha}$ a better substrate for the reductase(s) which reduces the Δ^5 double bond. The importance of the identification of the major urinary metabolite of 8-iso-$PGF_{2\alpha}$ is that it provides the basis for development of methods of assay for 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ as a means to obtain an integrated

assessment of total endogenous F_2-IsoP production in humans.

9. Biological actions of IsoPs

Because IsoPs are isomeric to cyclooxygenase-derived PGs, which exert potent biological activity, it was of considerable interest to explore whether IsoPs may not be simply markers of lipid peroxidation but also possess biological activity, in which case they may participate as mediators of oxidant injury. A unique structural feature of IsoPs is that, in contrast to cyclooxygenase-derived PGs, the side chains are predominantly oriented *cis* in relation to the prostane ring [5,6]. Thus, one of the compounds that would be predicted to be formed would be 8-iso-$PGF_{2\alpha}$. Because the E-ring IsoPs are formed by chemical rearrangement of the intermediate isoP endoperoxides, 8-iso-PGE_2 would also be expected to be formed from 8-iso-PGG_2. Recently, using high resolving HPLC approaches for purification of individual F_2-IsoPs from the complex mixture using radiolabelled 8-iso-$PGF_{2\alpha}$ as a marker, we found that 8-iso-$PGF_{2\alpha}$ is, in fact, one of the more abundant F_2-IsoPs that is produced in vivo [23]. Due to the fact that only these two IsoPs have been available in synthetic form for biological testing, our knowledge regarding the scope of biological actions of IsoPs is limited at present.

Because the levels of individual unmetabolized F_2-IsoPs in urine are very high, between 1–2 ng/ml [11], which, as discussed previously, may in part derive from local production in the kidney, we initially examined whether 8-iso-$PGF_{2\alpha}$ exerted any biological effects on renal function in the rat. Interestingly, it was found that 8-iso-$PGF_{2\alpha}$ was an extremely potent renal vasoconstrictor, reducing glomerular filtration rate and renal blood flow by 40–45% in the low nanomolar range [7,33]. During systemic infusion of 8-iso-$PGF_{2\alpha}$ that was associated with a fall in renal blood flow of $\simeq$ 50%, no alteration in systemic blood pressure occurred, suggesting a selective effect of this IsoP on renal vasculature. The primary site of action of 8-iso-$PGF_{2\alpha}$ in the glomerulus is constriction of the afferent renal arteriole, leading to a fall in glomerular capillary pressure [33,34]. In addition to its renal vasculature actions, 8-iso-$PGF_{2\alpha}$ has also been found to be a potent pulmonary artery vasoconstrictor in rabbits and rats and cause bronchoconstriction in the rat lung [35,36]. Interestingly, 8-iso-$PGF_{2\alpha}$ has also been shown to induce mitogenesis in vascular smooth muscle cells and induce endothelin-1 release from bovine aortic endothelial cells [34,37]. Of considerable interest was the finding that 8-iso-PGE_2 also is a potent renal vasoconstrictor, approximately equipotent with that of 8-iso-$PGF_{2\alpha}$ [13]. This was an unexpected finding because in most systems, cyclooxygenase-derived PGE_2 and $PGF_{2\alpha}$ have opposing biological effects, which has been attributed to the differences in ring structure. In particular, PGE_2 is a vasodilator whereas $PGF_{2\alpha}$ is a vasoconstrictor [38]. The finding that 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ are both potent vasoconstrictors in the renal vascular bed suggests that the stereochemistry of the side chains, rather than ring structure, may be an important determinant of the biological actions of IsoPs.

A series of investigations aimed at elucidating the mechanism by which 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2 exert their biological actions on vascular smooth muscle have led to provocative findings, namely that these IsoPs may exert their effects by interacting with a unique receptor. Initially we found that the renal vasoconstricting actions of these compounds could be abrogated by SQ29548, a thromboxane receptor antagonist, suggesting that these compounds interacted with thromboxane receptors [13,33]. Interestingly, however, when incubated with platelets, 8-iso-$PGF_{2\alpha}$ (10^{-6} and 10^{-5} M) caused only a shape change and at very high concentrations (10^{-4} M) only induced reversible but not irreversible aggregation [39]. 8-iso-PGE_2 caused a modest degree of irreversible aggregation of platelets from some individuals at concentrations of 10^{-5} and 10^{-4} M but in most, it only caused reversible aggregation at these concentrations [40]. In contrast, both IsoP's were more potent as antagonists of thromboxane receptor agonist-induced platelet aggregation. These findings, namely that these compounds acted primarily as antagonists of the thromboxane receptor in platelets, would not be consistent with these compounds interacting with thromboxane receptors unless the platelet and vascular smooth muscle thromboxane receptors were different. Although this would be one possible explanation for these findings, it is not very attractive owing to the fact that only a single thromboxane receptor gene has

been identified [41]. Splicing variants of the thromboxane receptor have been identified which appear to have similar ligand binding characteristics and phospholipase C activation but oppositely regulate adenylyl cyclase activity [42,43]. However, evidence suggests that 8-iso-$PGF_{2\alpha}$ does interact with either of these isoforms of the thromboxane receptor [44].

Another explanation for the above findings was the possibility that the IsoPs interacted with a unique receptor on vascular smooth muscle distinct from the thromboxane receptor. In order to explain the fact that the vascular effects of these compounds is abrogated by SQ29548, one would envision that this novel receptor must be structurally similar to the thromboxane receptor. Initial clues consistent with the presence of a receptor for IsoPs distinct from the thromboxane receptor was the finding that, whereas the IsoPs were more potent in inducing a functional response on vascular smooth muscle cells, e.g. phosphoinosotide turnover, than thromboxane receptor agonists, they were much weaker than thromboxane receptor agonists in displacing thromboxane receptor ligand binding [34]. For example, although U46619, a thromboxane receptor agonist, is significantly less potent than 8-iso-$PGF_{2\alpha}$ in inducing phosphoinosotide turnover in rat vascular smooth muscle cells, it is two orders of magnitude more potent than 8-iso-$PGF_{2\alpha}$ in displacing thromboxane ligand binding to the same cells. Preliminary data using radiolabelled binding studies is also consistent with the presence of a unique receptor for these IsoPs [37,45]. In these studies, low-affinity and high-affinity binding sites were found for 8-iso-$PGF_{2\alpha}$ both in vascular smooth muscle cells and endothelial cells and it was speculated that the former may represent binding to the thromboxane receptor and the latter binding to an 'IsoP receptor'. Although these data are consistent with the presence of a novel 'IsoP receptor', definitive proof must await the results of molecular approaches aimed at cloning this receptor.

10. Value of measuring IsoPs to assess oxidative stress status in vivo

As mentioned previously, most non-invasive methods developed to detect free radical injury in vivo have been found to be unreliable. However, a considerable body of evidence has been obtained that suggests strongly that measurement of IsoPs represents an important advance in our ability to assess oxidative stress status in vivo.

First, it is important to point out that IsoPs are specific products of lipid peroxidation. In this regard, however, it had been known previously that minute quantities of the F_2-IsoP, 8-iso-$PGF_{2\alpha}$, can be produced as a minor byproduct of the cyclooxygenase enzyme [46]. More recently, it was demonstrated that very small quantities of 8-iso-$PGF_{2\alpha}$ are formed by PGH synthase-1 during aggregation of human platelets in vitro and by PGH synthase-2 in human monocytes [47,48]. However, the amounts of 8-iso-$PGF_{2\alpha}$ formed by activated platelets and monocytes are only approx. 1/1000–2000th and 1/130–200th of the amount of thromboxane formed, respectively. Importantly, we originally demonstrated that the administration of high doses of cyclooxygenase inhibitors to normal humans does not suppress the intensity of the capillary GC peak in which 8-iso-$PGF_{2\alpha}$ elutes [7]. The failure of cyclooxygenase inhibitors to suppress levels of 8-iso-$PGF_{2\alpha}$ in normal volunteers has subsequently also been confirmed by others [24,49]. This indicates that the relative contribution of enzymatic generation of 8-iso-$PGF_{2\alpha}$ in vivo is inconsequential compared to the amounts formed non-enzymatically. However, it was speculated that in settings of enhanced platelet aggregation in vivo, the contribution of the cyclooxygenase to levels of 8-iso-$PGF_{2\alpha}$ could potentially become significant. However, results of a recent study does seem to bear out this concern [50]. Further, we have measured plasma levels of F_2-IsoPs by quantifying the intensity of the peak in which 8-iso-$PGF_{2\alpha}$ elutes from the GC column in a patient with unusually severe systemic mastocytosis associated with a dramatic overproduction of PGD_2 ($\approx$ 100-fold above normal). In this patient, the level measured was well within the normal range (unpublished data). Thus, even in pathologic situations associated with a profound increase in cyclooxygenase activity, enzymatic generation of 8-iso-$PGF_{2\alpha}$ remains insignificant in relation to the amounts formed non-enzymatically in vivo. Nonetheless, simply measuring one or more of the other F_2-IsoPs can provide a specific and reliable marker of free radical catalyzed lipid peroxidation because 8-iso-$PGF_{2\alpha}$ is the only F_2-IsoP that has

been shown capable of being produced by an enzymatic process.

Initial clues that measurement of IsoPs may provide a valuable approach to assess oxidative stress status in vivo emerged from some of the early studies that we carried out related to the discovery of these compounds [7]. Importantly, as mentioned previously, we can detect measurable levels of IsoPs in virtually every animal and human biological fluid and tissues that have been analyzed. This allows the definition of a normal range such that even small increases in the formation of IsoPs can be detected. Further, overproduction of IsoPs has been well documented to occur in settings of oxidant injury. For example, in our earlier studies we found that the formation of IsoPs increased as much as 200-fold above normal in two well established models of oxidant injury, namely the administration of CCl_4 to normal rats and the administration of the herbicide, diquat, to Se-deficient rats [7,9].

We then carried out studies correlating measurement of IsoPs with other established methods to assess lipid peroxidation, i.e. measurement of malondialdehyde (MDA) by the thiobarbituric acid reacting substances (TBARS) assay and measurement of mono-hydroxy derivatives of arachidonic acid [19,51]. In peroxidizing microsomes in vitro, the formation of MDA quantitatively far exceeded that of IsoPs. However, the fold-increases over baseline and the time-course of formation of both MDA and IsoPs were highly correlated. We then compared MDA and IsoPs as an index of lipid peroxidation in vivo by measuring these compounds in livers of rats which had been given CCl_4 to induce severe lipid peroxidation. Interestingly, levels of MDA increased less than 3-fold in the livers of CCl_4-treated animals, whereas the levels of F_2-IsoPs increased by as much as $\approx$ 80-fold [51]. The reason for the trivial increases in MDA compared to F_2-IsoPs in these animals is not understood but could be explained by rapid metabolism of MDA [51]. Even though the TBARS assay for MDA is well know to lack specificity [17], this aside, the large quantitative differences in the fold increases in MDA and IsoPs in CCl_4-treated rats indicates that the sensitivity of measuring IsoPs to detect lipid peroxidation in vivo far surpasses that of measuring MDA by the TBARS assay. Similarly, in another study, we found that the increases in levels of F_2-IsoPs measured in the livers of CCl_4-treated rats greatly exceeded increases in the levels of mono-hydroxy arachidonic acid derivatives in the liver [19]. These data indicate that the value of measuring of IsoPs to assess lipid peroxidation in vivo greatly exceeds that of other standard approaches used to quantify lipid peroxidation.

11. Use of measurements of IsoPs to explore the role of free radicals in the pathophysiology of disease in humans and animal models of disease processes

The fact that measurement of IsoPs appears to provide a very reliable index of lipid peroxidation in vivo provides us with a tool to assess the role of free radicals in the pathophysiology of human disease in a way that has not been possible previously. Some examples where measurement of IsoPs has provided new evidence for a role of oxidant stress in human disease and animal models of disease processes are summarized below.

11.1. Hepatorenal syndrome and acetaminophen poisoning

Our first ingress into the area of clinical investigation involved exploring the hypothesis that oxidant stress may play a role in the hepatorenal syndrome. Hepatorenal syndrome is defined as the onset of renal failure of unknown etiology in patients with severe liver disease. The pathophysiology of the renal failure has been attributed to intense renal vasoconstriction but the cause of the renal vasoconstriction remains poorly understood and may be multifactorial [52]. These patients frequently exhibit chronic endotoxemia and tissue hypoxia, an environment conducive for the generation of free radicals. Therefore, we measured circulating levels of F_2-IsoPs in 12 patients with the hepatorenal syndrome and appropriate control groups including normal volunteers, patients with chronic renal failure and normal liver function, and patients with severe and mild liver disease in whom renal function was normal. Circulating plasma concentrations of F_2-IsoPs were selectively increased a mean of 7.8-fold in patients with hepatorenal syndrome compared to the control groups ($P < 0.001$)

[53]. We also measured circulating concentrations of F_2-IsoPs in 10 patients with acute liver and renal failure associated with acetaminophen (paracetamol) overdose. In this group, plasma F_2-isoprostane concentrations were also increased a mean of 9.1-fold above normal ($P < 0.001$) (manuscript submitted). Whether the renal failure in these patients is due to direct nephrotoxicity from acetaminophen metabolites [54] and/or a variant of the 'conventional' form of hepatorenal syndrome is unclear. In three of these patients we explored the effect of infusing a single dose of superoxide dismutase on circulating levels of F_2-IsoPs. In all three patients, plasma levels decreased to $\approx 50\%$ of preinfusion levels between 30–60 min after administration of the agent, consistent with a superoxide-dependent oxidative process. Since the duration of the effect of the administration of a single dose of superoxide dismutase is very short due to its rapid elimination, the effect of the reduction of plasma concentrations of F_2-isoprostanes on renal function could not be assessed in this acute study.

These findings suggest a role for free radicals in the pathogenesis of this almost uniformally fatal disease. It is of great importance to mention that liver transplantation in these patients is usually associated with a return of normal or near-normal renal function. Unfortunately, many patients with hepatorenal syndrome succumb before a suitable liver donor can be found. However, our results form a rational basis to explore whether antioxidant therapy may be effective in preventing death during the bridging the interval between the onset of hepatorenal syndrome and identification of a suitable donor liver for transplantation in these patients. As previously discussed, both 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2 are potent renal vasoconstrictors. Although it remains to be proven, it is attractive to consider the possibility that these IsoPs are contributing to the renal vasoconstriction that characterizes this disorder. In addition, the finding that 8-iso-$PGF_{2\alpha}$ induces endothelin-1 release may help to explain the large increases in plasma endothelin-1 concentrations in the hepatorenal syndrome [37,55]. As noted previously, the vasoconstriction caused by these IsoPs can be abrogated by at least some thromboxane receptor antagonists. In this regard, we have a study currently underway to assess whether treatment of patients with hepatorenal syndrome with a thromboxane receptor antagonist is associated with an improvement in renal function; preliminary results from this study appear promising.

11.2. Scleroderma

The pathogenesis of the disease, scleroderma (systemic sclerosis) is unknown but recently the possibility that oxidant injury may be involved in the tissue damage in this disease was proposed [56]. Not only can free radicals stimulate fibroblast proliferation and fibrosis, which characterizes this disease, they can also inhibit the action of NO, resulting in enhanced vasoconstriction, another hallmark of scleroderma. To explore whether there was evidence of oxidative stress in scleroderma, we measured the excretion of F_2-IsoP urinary metabolites in 10 healthy controls and 8 patients with scleroderma (representing a wide spectrum of disease severity ranging from limited to diffuse disease) [57]. The urinary excretion of F_2-IsoP metabolites was elevated in scleroderma patients a mean of 280% above control ($P < 0.002$). Elevations were seen both in patients with limited and diffuse disease but the highest excretion of F_2-IsoP metabolites (483% above control) was seen in a patient with diffuse disease.

These data suggest a role for free radicals in the pathogenesis of scleroderma and provides a marker whose relationship to disease activity and disease therapy may be important. Further, these findings may also provide a rationale to explore whether antioxidant therapy may influence the natural course of this disease.

11.3. Oxidative injury in chronic cigarette smokers

Cigarette smoke contains a large number of oxidants [58]. Thus, it has been suggested that cigarette smoking may cause oxidative damage. Since oxidation of DNA and LDL may lead to cancer and atherosclerosis, respectively, oxidative injury to these important biomolecules by cigarette smoke may provide a mechanistic link between the enhanced incidence of cancer and atherogenesis in individuals who smoke [59,60]. In an attempt to assess whether there is evidence of oxidative stress in smokers, we measured levels of F_2-IsoPs both free in the circulation and esterified to plasma lipids and the urinary excretion of F_2-IsoP metabolites in 10 heavy smokers and

10 age- and gender-matched non-smokers. Levels of both free and esterified F_2-IsoPs were significantly elevated in smokers compared to non-smokers by a mean of 235% ($P = 0.02$) and 166% ($P = 0.03$), respectively [29]. Further, there was a high correlation between the urinary excretion of F_2-IsoP metabolites and plasma concentrations of F_2-IsoPs ($r = 0.97$, $P < 0.001$). Following 2 weeks of abstinence from smoking, levels of free and esterified F_2-IsoPs in plasma fell significantly by a mean of approx. 35%. These findings suggest strongly that cigarette smoking causes oxidative injury which may provide a causative link between smoking and the development of cancer and atherosclerosis.

11.4. Other disorders and animal models of disease processes

Enhanced production of IsoPs has also been reported to occur in association with several additional disorders and animal models of disease processes in which free radicals have been thought to play a role. For example, we have extensively studied animals in which severe liver injury was induced by administration of CCl_4 and the herbicide, diquat. In both of these animal models, profound increases in the formation of IsoPs occurs, indicative of marked lipid peroxidation [7,9,61]. Similarly, levels of IsoPs are increased in tracheobronchial aspirate fluid of premature infants exposed to increased oxygen concentrations [62]. There also seems to be a trend towards increased production of IsoPs with increasing age, data which may lend some support to the oxidation hypothesis of aging [22]. Organophosphate poisoning is associated with muscle endplate necrosis and increased levels of IsoPs esterified to muscle tissue was recently demonstrated in animals poisoned with organophosphates [63]. Further, administration of a lazaroid antioxidant suppressed both levels of IsoPs and protected against organophosphate-induced muscle necrosis, suggesting strongly that free radicals are importantly involved in the pathologic changes that occur in the muscle in association with organophosphate poisoning. Increased formation of IsoPs has also been demonstrated in settings of ischemia/reperfusion injury to both the liver and kidney [33,64]. Plasma levels of F_2-IsoP's have also been shown to correlate positively with severity of liver injury in experimental alcoholic liver disease in rats and urinary F_2-Isop excretion increases in a dose-dependent fashion following ingestion of alcohol in humans, data which provides strong support for the hypothesis that free radicals contribute to alcohol-induced liver injury [65,66]. Further, dietary iron overload has been shown to be associated with increased levels of F_2-IsoPs esterified to lipids in the livers of rats [67]. The anesthetic halothane can induce liver injury, especially under hypoxic conditions, which is thought to involve the production of free radicals via the reductive metabolism of halothane [68]. We recently demonstrated that in rats given halothane, even under normoxic conditions, increased levels of F_2-IsoPs are present esterified to hepatic lipids, indicative of free radical induced peroxidation of hepatic lipids [69]. On the other hand, measurements of IsoPs can also be useful in discounting a role for free radicals in pathology. For example, the administration of cyclosporine is frequently associated with a decrease in renal function, which has been speculated to involve free radical injury to the kidney [70]. However, we recently demonstrated that alterations in renal function in patients with rheumatoid arthritis being treated with cyclosporine were not associated with an increase in urinary IsoPs, suggesting that cyclosporine, at the doses administered to these patients, does not induce lipid peroxidation [71].

12. Summary

The discovery of IsoPs as unique products of non-enzymatic lipid peroxidation has been fortuitous in a many ways and has opened up numerous new avenues for investigation. First, the use of quantification of IsoPs as markers of oxidative stress status in vivo appears to be a major advance in our ability to explore the role of free radicals in the pathogenesis of human disease. One drawback related to this is the current lack of more facile and less expensive methods than mass spectrometry for the measurement of IsoPs. This greatly hampers the general use of measuring IsoPs by investigators in the free radical field. However, efforts are underway both in academia and industry to develop specific and reliable immunoassay methods for measurement of IsoPs. This most likely will lead to a prolific expansion of the use of

measurements of IsoPs to assess oxidative stress status in vivo both in animal models of oxidative injury and in humans. Another great need in the field of free radical medicine is information regarding the clinical pharmacology of antioxidant agents. Because of the evidence implicating free radicals in the pathogenesis of a number of human diseases, e.g. atherosclerosis, large clinical trials are planned or underway to assess whether antioxidants can either prevent the development or ameliorate the pathology of certain human disorders. However, data that accurately informs us regarding the most effective doses and combination of antioxidant agents to use in these clinical trials is lacking. As mentioned previously, administration of antioxidants can be shown to suppress the formation of IsoPs, even in normal individuals. Thus, measurement of IsoPs should provide a valuable approach to define the clinical pharmacology of antioxidants.

In addition to being valuable markers of oxidative stress, the IsoPs tested thus far possess interesting and potent biological activity. In this regard, the availability of additional IsoPs in synthetic form should significantly broaden our knowledge concerning the role of these molecules as mediators of oxidant stress. In addition, information regarding the nature of the receptor(s) that mediate the biological actions of IsoPs will be of considerable importance as it contributes to the development of specific antagonists or agonists of the biological actions of IsoPs.

Although considerable information has been obtained since the initial report of the discovery of IsoPs in 1990 [5,7], much remains to be understood about these unique molecules. With continued research in this area, we are confident that much new valuable information will be forthcoming which, in turn, will also likely open up additional important new areas for future investigation.

Acknowledgements

Supported by NIH grants GM42056, GM15431, and DK48837.

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